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# *Lactococcus lactis* GEM particles displaying pneumococcal antigens induce local and systemic immune responses following intranasal immunization

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## Abstract

The present work reports the use of non-living non-recombinant bacteria as a delivery system for mucosal vaccination. Antigens are bound to the cell-wall of pretreated *Lactococcus lactis*, designated as Gram-positive enhancer matrix (GEM), by means of a peptidoglycan binding domain. The influence of the GEM particles on the antigen-specific serum antibody response was studied. Following nasal immunization with the GEM-based vaccines, antibody responses were induced at systemic and local levels. Furthermore, different GEM-based vaccines could be used consecutively in the same mice without adverse effects or loss of activity. Taken together, the results evidence the adjuvant properties of the GEM particles and indicate that GEM-based vaccines can be used repeatedly and are particularly suitable for nasal immunization purposes.

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**Keywords:** Mucosal vaccine; *Lactococcus lactis*; Gram-positive enhancer matrix (GEM); *Streptococcus pneumoniae*; SlrA; PpmA; IgA1 protease

## 1. Introduction

In the past years considerable effort has been undertaken to develop mucosal vaccines and to understand mechanisms underlying mucosal immunity (for recent reviews see [1,2]). The nasal and oral routes are very attractive considering the ease of administration and the local immune response at the contact surface between the body and the outside world. In the future, mucosal vaccines may partly replace injectable vaccines provided that potent and relevant responses are elicited. Although encouraging results have been reported, improvements are still required, both in terms of efficiency and safety.

Most adjuvants with potency for mucosal immunization are derived from bacteria. They include the well-known cholera toxin and *Escherichia coli* heat-labile toxin as well as

their non-toxic variants, muramyl di-peptide [3], outer membrane proteins [4,5] and outer membrane vesicles [6] of e.g. *Neisseria meningitidis*. Whole cell *Bordetella pertussis* and *N. meningitidis* also provided nice results in animal models [7,8]. However, the use of pathogen-based vaccines/adjuvants is associated with safety concerns and the current research aims at finding alternatives. In this context, the lactic acid bacteria (LAB) are of special interest because they are generally recognized as safe (GRAS). Recombinant LAB expressing heterologous antigens have been successfully used for vaccination purposes. Immune responses could be elicited against bacterial and viral antigens [9,10]. Intranasal immunization led to humoral and cellular responses to HPV-16 E7 [11,10] and tetanus toxin [12]. Protection was demonstrated in animal models with a tetanus challenge using recombinant *Lactobacillus plantarum* [13,14], *Streptococcus gordonii* [15] and *Lactococcus lactis* [16,17] following different immunization routes (subcutaneous, nasal, oral). Because these recombi-

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nant LAB are suitable for mucosal immunization, they seem particularly useful for the development of vaccines against pathogens invading the body through the mucosal surface. This is illustrated by the recent work of Mannam et al. in which *L. lactis* cells expressing part of the virulent factor M provided protection in nasally immunized mice against a lethal nasal challenge with *Streptococcus pyogenes* [18]. Although *L. lactis* and other LAB are safe bacteria, their widespread use as recombinant strains in mucosal vaccinations may cause unwanted horizontal gene transfer of the recombinant DNA to other (micro)organisms.

In order to develop a safe and affordable mucosal vaccine delivery system we used a system that exploits killed non-recombinant *L. lactis* particles obtained by chemical pretreatment of whole bacteria with hot acid [19,20] (van Roosmalen et al., in press). These particles are referred to as Gram-positive enhancer matrix (GEM) and they constitute mainly of bacterial shaped peptidoglycan spheres that lack other intact cell wall components and intracellular material. Here, we studied their use as an antigen carrier and adjuvant in nasal immunizations. Antigens are attached to the surface of GEM particles by means of a peptidoglycan binding domain called the protein anchor (PA). The PA domain comes from a lactococcal enzyme and it binds with high affinity to peptidoglycans in a non-covalent manner [19,21] (Bosma et al., in press). Here, chimeric antigen-PA genetic constructs were prepared with three antigens of *Streptococcus pneumoniae* (*S. pneumoniae*): the putative proteinase maturation protein A (PpmA); the streptococcal lipoprotein rotamase A (SlrA); and the immunoglobulin A1 protease (IgA1p). *S. pneumoniae* can cause severe disorders as pneumonia, meningitis and septicaemia upon infection. The classical vaccine approach for this pathogen makes use of capsule polysaccharide-antigens. However, several candidates protein-antigens were isolated and promising results were obtained in animals models of infection by *S. pneumoniae* [20–24]. SlrA and IgA1p were recently demonstrated to be immunogenic in humans [25]. PpmA is able to induce antibodies with opsonophagocytic activity [26]. PpmA antibodies in humans are associated with a decreased risk of Otitis Media [27]. All three antigens are immunogenic, surface located and can induce antibodies in young children [25].

After expression in a suitable host to obtain secreted antigen-PA fusion proteins and binding to the GEM particles, the obtained vaccines were characterized in vivo studies. To demonstrate the adjuvant activity of GEM particles, serum antibody responses were measured after nasal immunization with different vaccine formulations of SlrA, with or without GEM particles. We also investigated the effect of a pre-existing anti-carrier immune response on the development of antigen-specific antibodies when two different vaccines were used consecutively in the same mice. We then prepared GEM-based vaccines with SlrA, PpmA and IgA1p and studied the local and systemic responses obtained after intranasal immunization.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*L. lactis* NZ9000 used for production of the GEM particles was grown in M17 broth (Oxoid, Haarlem, The Netherlands) at 30 °C or on M17 agar, both supplemented with 0.5% glucose (GM17). *L. lactis* PA1001 was used for the production of recombinant antigens and grown in GLS broth (Strik Special Additives, Eemnes, The Netherlands). *S. pneumoniae* D39 (NTTC 7466; Central public health laboratory, London, UK) and TIGR4 [28] were used for ELISA on whole bacteria and grown in Todd–Hewitt broth (Oxoid) supplemented with 0.5% yeast extract at 37 °C.

### 2.2. Vaccine preparation

The plasmids pPA32, pPA162 and pPA152 were used to express and secrete the recombinant fusion proteins PpmA-PA, SlrA-PA and IgA1 protease-PA (IgA1p-PA), respectively. Construction of the plasmids is described elsewhere (Audouy, manuscript in preparation). Lactococcal GEM particles were produced as described before [20,21]. Culture supernatants containing the fusion recombinant proteins were concentrated with a VivaFlow (Vivascience VivaFlow200, 10,000 Da cut-off). Binding of antigens was achieved by mixing the concentrates with GEM particles under gentle agitation for 30 min at room temperature. The amount of bound antigen-PA was estimated using coomassie brilliant blue (CBB) stained gels and comparison to bovine serum albumin (BSA) protein standards. Monovalent vaccines consisted of  $2.5 \times 10^9$  GEMs and contained 6 µg IgA1p-PA, 17 µg PpmA-PA or 73 µg SlrA-PA, per dose. The trivalent vaccine was prepared by mixing the three monovalent vaccines in a ratio 1:1:1 and thus, contained a third of the amount of antigen present in the monovalent vaccines for each antigen.

### 2.3. Production and purification of his-tag fusions

SlrA-His (pET11-SlrA), PpmA-His (pET11-PpmA) and IgA1p-His (pET11-IgA1 protease) with a C-terminal his-tag [25] were produced in *E. coli* BL21(DE3) using IPTG induction and purified by his-tag isolation for coating of ELISA plates.

### 2.4. Immunization and sample collection

Outbred female CD-1 mice (6 weeks old) were purchased from Harlan (Gannat, France). Mice were maintained under specified pathogen-free (SPF) conditions and received water and food ad libitum. All animal experiments were performed with approval of the Animal Experimentation Committee of the University of Groningen (Groningen, The Netherlands). Intranasal immunization was performed under light isoflurane inhalation anaesthesia. Mice were held on their backs

and 20  $\mu$ l of vaccine or control was gently applied onto the nostrils using a standard research pipet. Complete immunization consisted of three doses given at 10-day intervals, each containing  $2.5 \times 10^9$  GEM particles. At termination, blood samples were collected by cardiac puncture and supplemented with heparin. After centrifugation, plasma was stored at  $-20^\circ\text{C}$  until use. A small incision was made in the trachea for insertion of a small tube connected to a syringe to collect nasal and lung lavages. Lung washes were performed by injection of PBS into the lung followed by aspiration of the lavage fluid. Nasal washes were performed by aspiration of PBS and ejection of the lavage fluid through the nostrils. Nose and lung washes were collected, respectively, in 0.5 ml and 1 ml PBS containing protease inhibitor (Complete Protease Inhibitor Cocktail, Roche, Almere, The Netherlands). Samples were kept at  $4^\circ\text{C}$  until testing on the following day.

### 2.5. Detection of specific antibodies by ELISA

To determine the concentrations of specific anti-PpmA, -IgA1p, and -SlrA IgGs and IgAs an ELISA procedure was used. Briefly, high-binding capacity microtiterplates (Greiner, Haarlem, The Netherlands) were coated with SlrA-His (0.2  $\mu$ g/well), PpmA-His (0.2  $\mu$ g/well) or IgA1p-His (1  $\mu$ g/well) in 0.05 M carbonate buffer (pH 9.6) overnight at  $4^\circ\text{C}$ . Plates were washed with PBS (pH 7.4) with 0.02% Tween 20, then incubated 1 h with 1% BSA in PBS/Tween. Sera were diluted appropriately and added to the plates in three-fold dilutions and incubated for 2 h at room temperature. After washing, the alkaline phosphatase secondary antibody directed to mouse IgG-Fc or mouse IgA (Sigma, Zwijndrecht, The Netherlands) was incubated for 1.5 h at a dilution of 1:5000. Colorimetric reaction was obtained by addition of *p*-nitrophenyl phosphate substrate (Sigma) diluted in 0.05 M carbonate buffer (pH 9.6) supplemented with 1  $\mu$ M  $\text{MgCl}_2$ . The enzymatic reaction was stopped with NaOH and absorption was measured at 405 nm. Concentrations were calculated from a calibration curve made with purified mouse IgG (Sigma). IgG antibodies against whole *S. pneumoniae* bacteria were evidenced following the same ELISA protocol. The plates were coated with heat-inactivated bacteria ( $10^7$ /well) in carbonate buffer overnight at  $4^\circ\text{C}$ .

### 2.6. Immunoblotting

The level of serum antibodies against GEM components was determined by Western blotting. GEM particles were used untreated or treated with lysozyme to break down the cell wall. For lysozyme treatment, GEM particles were incubated for 1 h at  $55^\circ\text{C}$  in lysis buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 0.1 mg lysozyme). A protein gel was run with the samples, containing the equivalent of  $5 \times 10^8$  GEM particles per lane, and Western blots were incubated with a 100 $\times$  dilution of pooled sera from mice which received no vaccine (pre-immune), three doses of GEM vaccine ( $3 \times$  GEM) or six doses of GEM vaccine ( $6 \times$  GEM). The blot was

incubated with anti-mouse IgG alkaline phosphatase conjugate (Sigma) as second antibody and stained with NBT and BCIP using standard procedures.

## 3. Results

### 3.1. Formulation of the vaccine

The ability of the GEM particles to act as carrier and adjuvant was tested with the pneumococcal antigen SlrA. Only background levels of SlrA-specific IgGs were measured when purified SlrA was administered in the absence of any adjuvant (Fig. 1) and two mice in this group ( $n=6$ ) remained sero-negative. When SlrA was bound to or mixed with the GEM particles, high levels of serum IgGs were produced (significantly higher compared to levels obtained with purified SlrA ( $P$ -values  $<0.005$ )). The highest levels were obtained when SlrA was attached to the GEM particles. There was no significant difference ( $P=0.44$ ) between the bound and mixed groups and all mice in these groups ( $n=6$ ) showed sero-conversion. Interestingly, when the GEM particles were administered 24 h prior to antigen administration, still an increase in IgG production was found compared to immunization with the antigen alone ( $P<0.05$ ). However, the IgG concentration was in average half of the concentration obtained when the antigen and the particles were given at the same time. The ability of the GEM particles to increase the systemic antibody response against SlrA clearly demonstrates their natural adjuvant properties.

### 3.2. Effect of preexisting anti-carrier antibodies

We evaluated the effect of a preexisting antibody response against the GEM particles and protein anchor on a subsequent immunization with a GEM-based vaccine. Four groups

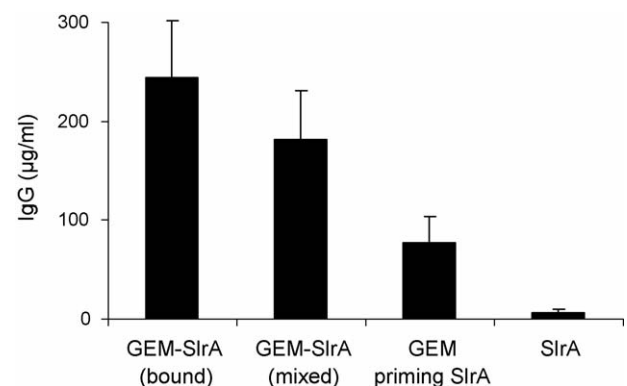


Fig. 1. Concentrations of anti-SlrA IgG antibodies in serum after three nasal immunizations with SlrA in different formulations: GEM particles with bound protein anchor-SlrA fusion product (GEM-SlrA bound), GEM particles mixed with purified SlrA (GEM-SlrA mixed), GEM particles administered 24 h prior administration of purified SlrA (SlrA GEM priming) or purified SlrA alone. Bars represent average concentrations per group + S.E.M. ( $n=6$ ).

Table 1  
Immunization schedule for consecutive immunizations and specific IgG concentrations ( $\mu\text{g/ml}$ ) after the first immunization series

Group	First immunization series	Specific IgG ( $\mu\text{g/ml}$ )			Second immunization series
		PA	SlrA	PpmA	
1	PBS	0	0	0	GEM-PpmA
2	GEM-SlrA	$68 \pm 19$	$223 \pm 50$	0	GEM-PpmA
3	PBS	0	0	0	GEM-SlrA
4	GEM-PpmA	$78 \pm 39$	0	$207 \pm 103$	GEM-SlrA

Results are averages/group  $\pm$  S.E.M. ( $n=6$ ).

of mice ( $n=6$ ) received first a series of three nasal immunizations according to the schedule in Table 1. A second immunization series was started three months later. One week after the first immunization series and one day before the second series, serum samples of all mice were analyzed for the presence of SlrA, PpmA and PA specific IgGs. At both time points high concentrations of circulating IgG antibodies against the pneumococcal antigens and PA could be detected in the two groups that received GEM-based vaccines (Table 1). We found no decrease in these antibody levels during the three month intervals between both immunization series (data not shown). No specific antibodies were found in the mice treated with PBS. Mice first immunized with GEM-SlrA received a second series of nasal immunizations with GEM-PpmA. Similarly, mice first immunized with GEM-PpmA received a second series of nasal immunizations with GEM-SlrA. Mice pretreated with PBS received either a GEM-PpmA or a GEM-SlrA vaccine (Table 1). Similar anti-PpmA (Fig. 2A) and anti-SlrA (Fig. 2B) IgG levels were raised regardless of the pretreatment that the mice received during the first immunization, i.e. a different GEM-based vaccine or PBS. In addition, the development of specific IgGs followed the same kinetics in PBS and vaccine pretreated mice. Already after a single administration measurable amounts of anti-PpmA and anti-SlrA IgGs were circulating in the blood. IgG levels increased upon a second and third administration which is characteristic of an adaptive response and indicates that immunological memory was developed. When a first immunization series with GEM-PpmA was started in mice at the age of 6 weeks or 22 weeks (group 1 and 4 in Table 1), the concentrations of specific IgG antibodies 10 days after the third dose were  $207 \mu\text{g/ml}$  and  $229 \mu\text{g/ml}$ , respectively. A similar observation was made for the GEM-SlrA vaccine, with average concentrations of  $223 \mu\text{g/ml}$  in the young and  $163 \mu\text{g/ml}$  in the older mice. Serum IgA levels for PpmA and SlrA were also measured after all immunizations. Systemic anti-PpmA IgA concentrations were  $0.61 (\pm 0.27) \mu\text{g/ml}$  and  $1.9 (\pm 0.63) \mu\text{g/ml}$  when the GEM-PpmA vaccine was given in naïve or preimmunized mice, respectively. Systemic anti-SlrA IgA concentrations were  $11.9 (\pm 5.9) \mu\text{g/ml}$  and  $5.3 (\pm 1.7) \mu\text{g/ml}$  when the GEM-SlrA vaccine was given in naïve or preimmunized mice, respectively. The differences were not statistically significant ( $P=0.09$  and  $0.3$  in the case of PpmA and SlrA, respectively).

Anti-PA antibodies were raised upon immunization with the GEM-PpmA and GEM-SlrA vaccines as shown in Table 1 and specific IgG levels were comparable in both cases. However, anti-PA IgG levels were about a factor 3 lower compared to the levels of IgGs induced against the pneumococcal antigens.

To evaluate the immune response against the components of the empty GEM carrier particle, Western blots containing GEM and lysozyme-treated GEM were incubated with the sera of mice which received three doses of GEM vaccine ( $3 \times$  GEM) or six doses of GEM vaccine ( $6 \times$  GEM).

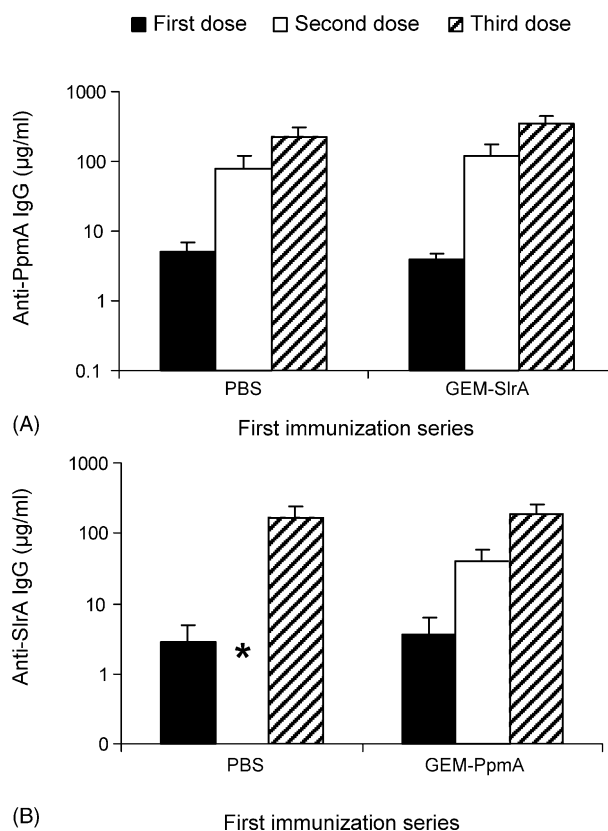


Fig. 2. Consecutive nasal immunizations with GEM-based vaccines carrying different antigens. Specific anti-PpmA (A) and anti-SlrA (B) IgG concentrations in serum after the first, second and third dose of the second immunization series of the GEM-PpmA (A) or GEM-SlrA (B) vaccine. The first series of immunizations contained either PBS or a GEM-based vaccine, as indicated on the x-axis. Bars represent average concentrations per group  $\pm$  S.E.M. ( $n=6$ ); (\*) not determined.



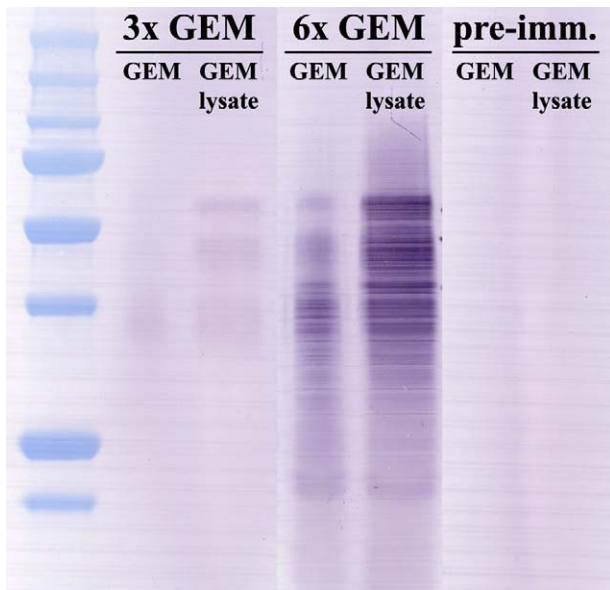


Fig. 3. Antibody response against the GEM carrier detected by immunoblotting. The GEM proteins on the blot were either released from intact GEM particles or from lysozyme treated GEM particles (lysate). The blots were incubated with a 100 times dilution of pooled sera from mice that received no vaccine (pre-immune), three doses of GEM-based vaccine (3 × GEM) or six doses of GEM-based vaccine (6 × GEM). The marker in the first lane contains proteins with sizes from top to bottom of 250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa and 20 kDa.

Preimmune serum was used as a control (see Fig. 3). After three immunizations with GEM-based vaccines, the antibody response against the carrier is very low. After six immunizations, however, antibodies were detected against the proteins that are still present in low amounts in the GEM particles. These remaining proteins were best visualized in the lane with the lysozyme-treated GEM sample, suggesting that these are intracellular proteins. This was corroborated by the fact that no antibody binding to intact GEM particles could be demonstrated in immunofluorescence assays when GEM particles had been incubated with serum of mice that received six doses of GEM-based vaccine (results not shown).

Taken together, these results clearly indicate that pre-existing immune responses against the carrier and a given antigen-PA fusion are not detrimental for the induction of systemic and mucosal immune responses against a different antigen-PA fusion using the same delivery system and the same immunization route. Moreover, the immune response measured was not dependant of the age of the mice at the time of priming.

### 3.3. Local antibody response

We then examined the local antibody response after nasal immunization against three pneumococcal antigens. SlrA-, PpmA- and IgA1p-PA fusion proteins were bound individually to the GEM particles and mice were immunized with a single monovalent vaccine. Specific IgA antibodies, the major characteristic of mucosal immunity, were measured

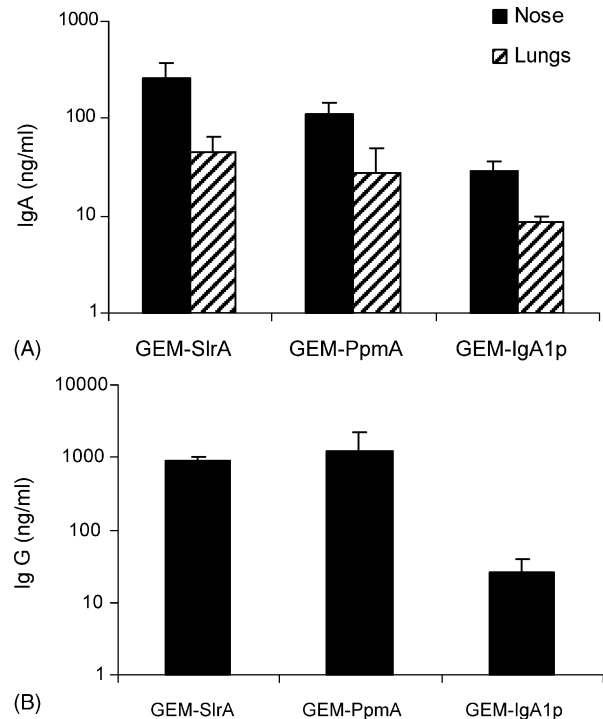


Fig. 4. Specific anti-PpmA, -IgA1p and -SlrA IgA (A) concentrations in nasal and lung washes after nasal immunization with GEM-based monovalent vaccines. Specific anti-PpmA, -IgA1p, and -SlrA IgG (B) concentrations in lung washes, after nasal immunization with GEM monovalent vaccines. Bars represent average concentrations per group + S.E.M. ( $n = 3-5$ ).

in the nasal and the lung secretions (Fig. 4A). All antigens induced IgA production at both locations. However, IgA concentrations against SlrA and PpmA were significantly higher than the IgA concentration against IgA1p. In the lung washes, IgG antibodies were the most abundant immunoglobulin class (Fig. 4B) with much higher concentrations compared to the antigen-specific IgA antibodies. The anti-IgA1p IgG level was lower compared to levels of IgG against the two other antigens, consistent with IgA production patterns in the nose and lungs. From these results we can conclude that the mucosal immune system was efficiently triggered upon nasal immunization with the GEM-based vaccines and reacted with adapted effector responses.

### 3.4. Systemic antibody response

The vaccine-induced systemic response was evaluated by measuring specific IgG levels in serum when the antigens were administered individually (Fig. 5A) or all together (Fig. 5B). Each antigen induced the production of circulating specific antibodies when given as a monovalent GEM-antigen vaccine (Fig. 5A). Concentrations of anti-SlrA (277  $\mu$ g/ml average) and anti-PpmA (207  $\mu$ g/ml average) IgGs were comparable, while anti-IgA1p IgG levels were much lower (7  $\mu$ g/ml average). These concentrations of IgG antibodies reflect the trend observed at the mucosal level. The three antigens were then administered together in a trivalent vaccine

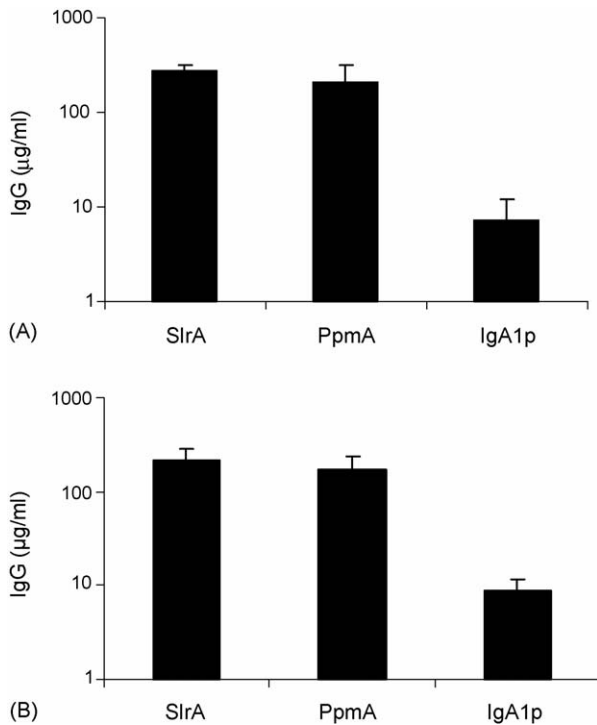


Fig. 5. Specific anti-PpmA, -IgA1p and -SlrA IgG concentrations in serum, after nasal immunization with GEM-based monovalent vaccines (A) or trivalent vaccine (B). Bars represent average concentrations per group + S.E.M. (A,  $n = 5$ ; B,  $n = 10$ ).

which consisted for each antigen of a third of the monovalent vaccine. Although only a third of each antigen dose from the monovalent vaccines was present in the trivalent vaccine, the IgG response developed against each antigen after nasal immunization was similar to the response obtained with the monovalent vaccines (Fig. 5B). This observation implies that the amount of antigens bound to the GEM particles was above the optimum for the induction of the IgG response or that the combination of the vaccines had a positive effect on this response. No negative interactions between the antigens were observed. Interestingly, the serum from mice immunized with the trivalent vaccine did not only recognize the purified antigens but also contained antibodies able to bind intact *S. pneumoniae* bacteria, strains D39 and TIGR4 (Table 2). Taken together, these data show that

Table 2  
ELISA on whole bacteria

Coating on ELISA plate	Serum from mice immunized with	
	PBS	Trivalent vaccine
D39	0.30	0.85
TIGR4	0.25	0.77
Trivalent vaccine	0.12	2.70

Pooled serum (diluted 1/100) from mice nasally immunized with PBS or the trivalent GEM vaccine was assayed for its ability to recognize *S. pneumoniae* strains D39 and TIGR4 and the GEM-based trivalent vaccine coated on a microtiter plate. Optical density values from a standard ELISA test are shown (OD measured at 405 nm).

mucosal vaccination with GEM-based vaccines results in a systemic humoral response. In addition, the GEM system can be used to immunize against multiple antigens simultaneously and more specifically, SlrA, PpmA and IgA1 protease can be combined in a trivalent vaccine.

#### 4. Discussion

In the work presented here we characterize a vaccine delivery and adjuvant system based on killed non-recombinant *L. lactis* particles (GEM) displaying antigens on their surface by means of a peptidoglycan binding domain. Our approach differs in several aspects from the use of recombinant lactic acid bacteria producing heterologous proteins as antigens. However, as with live *L. lactis* cells, the GEM particles possess strong adjuvant properties [29]. Further comparison of both strategies is difficult because of the differences in antigens used, in antigens concentrations and in numbers of bacterial cells/GEM particles delivered. Several authors working with live recombinant LAB, showed that stronger responses were mounted when the antigen was bound to the membrane/cell-wall compared to secreted or intracellularly located antigen [10,16,30]. Usually these studies neglected the effect of antigen dose because a standard number of bacteria were given for immunization and recombinant bacteria expressed different amounts of antigen according to the antigen destination [10,30]. In our study the amount of antigen in each formulation was adjusted quite accurately. The difference we observed between bound or mixed was less pronounced (statistically not significant) than in the above-mentioned studies. An explanation might be that we used a relative high dose of the SlrA antigen. Differences between mixed and bound may become significant when lower amounts of antigen are administered. Further investigations are required to understand the effect of binding to the GEM through the PA domain on the development of an immune response. The observation of enhanced antibody responses to SlrA when the GEM particles were given 24 h prior to the antigen implies that complex factors play a role. The GEM particles seem to place the initiation site of the immune response into a 'receptive' state for the antigen and this could be the result of inducing the expression of co-stimulatory molecules on antigen-presenting cells or the release of cytokines as TNF- $\alpha$ . These issues should be addressed not only with SlrA but also with other antigens to rule out an antigen-related effect. Regardless of the effect of attachment of the antigen to the carrier on the immune response, the strong binding of antigen-PA fusion proteins to the peptidoglycan layer of the GEM particles provide an efficient and rapid purification method for the antigen, thereby facilitating easy formulation of the vaccine.

Low levels of IgG were developed against intracellular protein remnants in the GEM particles, which is consistent with the finding of others that *L. lactis* cells have a low intrinsic immunogenicity [16,17]. In addition, these antibodies hardly recognize intact GEM particles. After six adminis-

trations of the GEM vaccine antibodies react with GEM proteins in the Western blot. The SDS–PAGE treatment without lysozyme does lyse some of the GEM particles. Therefore, the proteins that light up in the Western blots are likely to be intracellular proteins. This was corroborated by the fact that reaction of the antiserum with the intact GEM particles was not detected using immunofluorescent microscopy. Consequently, the antiserum does not contain antibodies that recognize surface components of the GEM particles. We also showed that the preexisting antibody responses to the GEM-PA complex did not hamper the development of systemic and mucosal antigen-specific antibody responses upon nasal vaccination. Several studies have addressed the question of preexisting immunity against the CT/CTB adjuvant in combination with antigens of different nature, e.g. dextran [31], influenza virus [32], and capsular polysaccharide [33]. In the latter case, divergent findings were made, as either increase or inhibition of the anti-CPS response was seen after priming with the toxin [33,34]. Wu and Russell showed with a bacterial protein antigen that the development of salivary IgA and serum IgG responses were largely unaffected by a pre-immunization against CT when a CT-antigen conjugate was used for subsequent immunization [35]. Similarly, our data showed that antigen-specific systemic antibody levels developed to a similar level with or without preexisting anti GEM-PA antibodies. Others showed that serum and mucosal IgA anti-polysaccharide were reduced when a anti-CT response was previously induced intranasally [31,33]. However, this effect was reversed when the immunization was done three months after the anti-CTB priming, while anti-CTB titers were still high [31]. Bergquist et al. [31] suggested that local memory T-cell specific for the carrier/adjuvant could help the initiation of the mucosal response. Indeed these authors saw a slight increase in IgA anti-dextran levels in the lungs when mice were vaccinated with dextran-CTB conjugate three months after CTB priming. Measurement of the local immunoglobulins in our experimental set-up may provide useful information to understand mechanisms of inhibition/enhancement. In contrast to the referred studies where the pre-immunization was performed with the adjuvant only, we used two complete vaccines successively, which better reflects a practical situation. In addition, administration schedules appear to be a critical parameter and although we used a different vaccine delivery system, the same may hold true in our case. These complex and, in our view, critical issues deserve more investigations.

Lactic acid bacteria seem promising organisms for vaccination against *S. pneumoniae*. We showed in the present work expression of three pneumococcal antigens in *L. lactis*. Expression of pneumococcal surface antigen A (PsaA) and pneumococcal surface protein A (PspA) was already reported in *L. casei* [36] and *L. lactis* (Leenhouts, manuscript in preparation). Furthermore, IgA and IgG antibodies were developed against these antigens when fused to PA and displayed on the lactococcal GEM particles for nasal immunization. The IgA1p antigen induced much lower antibody responses com-

pared to SlrA and PpmA. An obvious explanation is that the mice received a considerable lower amount of IgA1p per dose than that for SlrA or PpmA. However, further investigation is needed to reveal whether the dose or the immunogenicity of IgA1p is the crucial factor on this point. The IgA1p present in the GEM vaccines is a truncated antigen, lacking enzymatic activity, which excludes cleavage of nose IgAs by the antigen.

In summary, the work presented in this paper addressed critical aspects of nasal immunization with GEM-based vaccines. We showed that the IgG antibody response induced against a pneumococcal antigen was dramatically increased by the GEM particles compared to antigen alone. Two subsequent immunization series with different GEM-based vaccines could be performed without affecting the systemic antibody response to a specific antigen, compared to single immunization against the same antigen. Furthermore, we demonstrated that GEM-based vaccines elicited mucosal and systemic antibody responses against three pneumococcal antigens following nasal immunization, without additional adjuvants. Taken together, these results clearly illustrate the potential of the GEM-system for the development of mucosal vaccines against *S. pneumoniae* in particular and other pathogens in general.

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